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14. ABSTRACT

(Taken From Initiating PI Report)
Prostate cancers that recur after so-called androgen ablation therapy ('CR-CaP') are typically more aggressive, more likely to spread to local lymph nodes and bones, and less likely to respond to second-tier treatments, and therefore, contribute to significantly decreased patient survival. We posit that enzymes called Src-family kinases (SFK) are required for the progression to CR-CaP, and thus, targeting these enzymes should prevent CR-CaP formation to suppress their growth. We will use animal models of human and mouse CR-CaP in conjunction with genetic and biochemical experiments to show that SFK are critical to the formation of CR-CaP, and thus, are therapeutically targetable using SFK-specific drugs. Our important pre-clinical studies on the critical role played by SFK in CR-CaP disease will serve as the foundation to establish immediate clinical trials in which CaP patients are treated with drugs such as KX2-391 at the commencement of androgen-deprivation therapy.

15. SUBJECT TERMS Src, androgen receptor, castration recurrent, human xenografts, src and akt isoforms

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Introduction:

From Initiating PI:

We are studying the role of Src-family kinases (SFK) in promoting castration-recurrent prostate cancer (CR-CaP) using genetic and pharmacological approaches along with several animal models of CR-CaP. Our synergistic collaboration is based the expertise of the initiating PI (Gelman) in the molecular signaling of SFK in cancer progression, combined with the expertise of the partnering PIs in the CWR22 and TRAMP CR-CaP mouse models (Mohler and Smith, respectively), and in the role of neuroendocrine cells (NE) in the progression of CR-CaP (Smith).

Body:

The initial role of my laboratory in the *Synergistic Research Program* was to provide expertise in NE-differentiation in animal models of prostate cancer, and to provide models for *in vivo* therapeutic evaluation of inhibitors *of src* family kinases *(SFKs)* in primary xenografts of human prostate cancer as a preclinical model. Our experimental studies were to be performed in primary cultures of prostate endothelial cells and primary xenografts of human prostate cancer tissue established from fresh surgical specimens of radical prostatectomy tissue. Importantly, this pre-clinical model is unique for analysis of the effects of SFK inhibitors on the individual prostate cellular compartments located within a human tissue microenvironment, including a human microvasculature. With our characterization of the presence of the individual SFK isoforms in primary cultures of human prostate endothelial cells during Year 1, the experimental emphasis of Year 2 has been the development of biological models for analysis of the role of specific SFK isoforms in human prostate endothelial cells, and for modeling the effects on prostate endothelial cells and the adjacent cancer epithelium of treatments that target SFKs.

The vasculature in the TRAMP tumor model of prostate cancer, and the CWR human prostate cancer xenograft model, is of host (mouse) origin. Importantly, the rodent prostate endothelial cells in these tumors are fundamentally different from the endothelial cells in human prostate cancers in that they do not express AR. Consequently, the experimental efforts in our portion of the *Synergistic Research Program* are focused on validation of human endothelial cell models that allow evaluation of the role of SFKs in the transport and metabolism of androgens, and as potential organ specific targets for systemic treatment with SFK inhibitors, in clinically relevant models. The human model systems are: 1) primary cultures of human prostate endothelial cells harvested from fresh surgical specimens; and 2) primary xenografts of fresh prostate tissue transplanted intact to immunocompromised hosts implanted with a source of testosterone to provide serum levels of androgen comparable to humans. The goal of this project is to evaluate the differential effect of inhibitors of SFKs on the homeostasis and angiogenic potential of prostate endothelial cells in castration-recurrent cancer in the androgen-deprived prostate. Our hypothesis is that the role of endothelial cells in the overall response to chemotherapy has been overlooked, even though endothelial cells represent a/the key barrier to the uptake of chemotherapeutic agents and circulating androgens, and that the state of the endothelial barrier may dramatically affect the progression to castration recurrent disease. Importantly, a competing hypothesis proposes that “vascular normalization” of tumor vasculature back to an intact, functioning endothelial barrier may portend a more effective and predictable response to systemic chemotherapy. Consistent with this hypothesis is fact that the endothelial cells are the first cells in the prostate to encounter systemically administered SFK-inhibitors, and recent studies that demonstrate that SFKs play a critical role in the trans-cellular transport of serum components through the endothelial cells, thereby affecting both the survival/proliferation of the endothelial cells as well as their transport/barrier functions. Therefore, understanding the response of prostate endothelial cells to SFK-inhibitors may provide valuable insight into their ultimate availability to prostate cancer epithelial cells, both androgen-stimulated and castration-recurrent, as well as predicting the importance of SFKs in endothelial cells as chemotherapy targets relative to solely targeting SFKs in cancer epithelial cells.

Lastly, the models of primary cultures of human prostate endothelial cells, and primary xenografts of human prostate cancer tissue, that can be established from multiple prostate cancer patients, provide powerful models for evaluation of the mechanistic role of SFKs, and their individual responses to SFK-inhibitors, using both chemotherapeutic modalities and isoform-specific shRNAs. Furthermore, these models allow investigations across a spectrum of patient tumors, not only for a single clonal cancer cell line.

In the current year, two lines of experimentation were initiated: 1) studies to evaluate the SFK-dependent, caveolae-mediated, response to paracrine signals using primary cultures of human prostate endothelial cells; and 2) studies to evaluate the effect of SFK-inhibitors on human angiogenesis in primary xenografts of human prostate tissue.

a) Src-mediated responses to systemically available signals:

Gene expression array analysis was conducted on primary cultures of human prostate endothelial cells cultured in the presence of androgen to determine if the essential components for SFK-mediated signaling were present. Table 1 shows that the genes coding for the proteins that are integral players in the receptor-initiated, caveolae-based signaling cascades were expressed in human prostate endothelial cells in primary culture, including: CD47 (thrombospondin-1 receptor), thrombospondin-1, caveolin-1, KDR (VEGF-R2), PI3K, HSP90, AKT and AR. It was unexpected that the mRNA for eNOS, a key generator of paracrine signals in response to membrane receptor activation, was expressed at low levels. It is not clear how this translates into eNOS protein levels in the cell, or if a low level of expression can compromise this critical signaling mechanism in the cultured cells. The level of expression of the individual SFK isoforms reported in last years progress report are included in the table to verify that expression of the YES, FYN and SRC isoforms is maintained in primary culture. The majority of the genes critical for caveolae-mediated signaling, including SFK and Akt, are expressed at levels greater than the median level of expression for all genes represented on the expression array.

Table 1. RELATIVE EXPRESSION OF GENES ASSOCIATED WITH SRC KINASE ACTIVITY IN PRIMARY CULTURES OF HUMAN PROSTATE ENDOTHELIAL CELLS

Gene	Description	Relative Expression
SRC	v-SRC sarcoma viral oncogene homolog	78.2
FYN	FYN oncogene related to SRC, FGR, YES	986.8
YES1	v-YES-1 Yamaguchi sarcoma viral oncogene	2056.9
LYN	v-YES-1 Yamaguchi sarcoma viral related oncogene	334.1
AKAP12	A kinase anchor protein (gravin 12 or SSeCKS)	4170.9
AKT1	v-AKT murine thymoma viral oncogene homolog1	104.4
AKT2	v-AKT murine thymoma viral oncogene homolog2	157.3
AKT3	v-AKT murine thymoma viral oncogene homolog3	2276.9
eNOS	Endothelial Nitric Oxide Synthase	87.8
CD36		66.0
CD47		555.0
Caveolin-1	Caveolin-1	14,200.0
KDR	Vascular Endothelial Growth Factor Receptor 2	6407.3
FLT-1	Vascular Endothelial Growth Factor Receptor 1	507.6
PDGF-RA	Platelet Derived Growth Factor Receptor	7.8
THBS1	Thrombospondin-1	25453.3
PI3K	Phosphotydal-inositol Kinase III	23.1
HSP90	Heat Shock Protein 90	7750.3
AR	Androgen Receptor	535.5
HIF-1	Hypoxia Inducible Factor 1	589.9

Relative Expression Level: upper 10% (1923.2); 25% (416.2); 50% (67.4); 75% (11.8)

Previously, primary cultures of human prostate endothelial cells were utilized to study the role of androgen in the stimulation of angiogenesis as measured by endothelial tube formation in Matrigel. This assay of angiogenesis evaluates treatment effects on chemotaxis, migration and cellular attachment, and is independent of endothelial cell proliferation. During this year, we evaluated the effect on endothelial tube formation by HUVEC of shRNAs specific for inhibiting expression of each of the four major isoforms of SFKs. The HUVEC cell line was employed as a surrogate for primary cultures of prostate endothelial cells to establish experimental conditions for analysis of tube formation before implementation of the assay using the more demanding primary culture-based system. The lentivirus vectors carrying the shRNAs for the four SFK isoforms were obtained from Dr. Gelman (PI of Synergistic Award) through his capacity as Director of the shRNA Core Resource at RPCI.

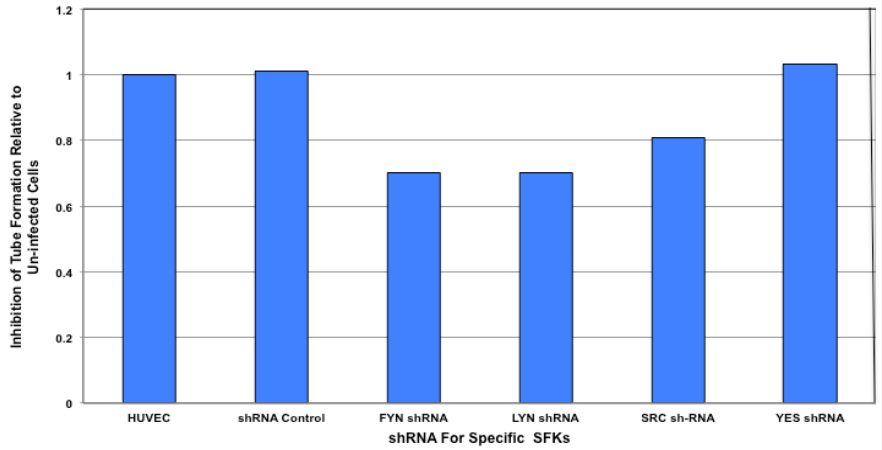


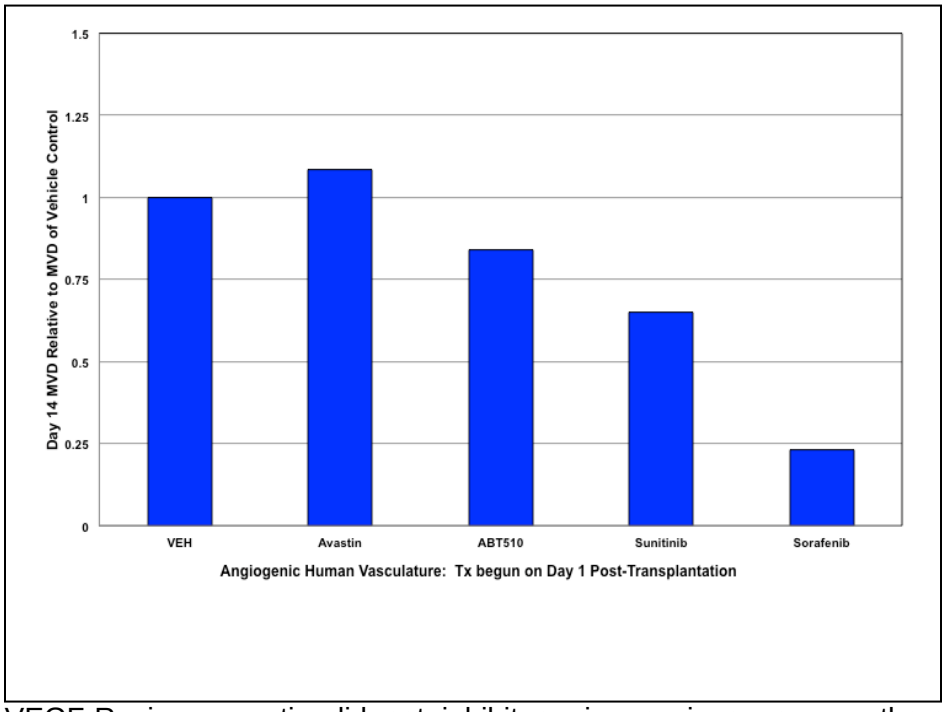
Figure 1. Inhibition of Endothelial Tube Formation by SFK isoform specific shRNAs

Figure 1 demonstrates that the individual isoforms of the SFKs differentially regulated endothelial tube formation by HUVEC (all assays done in the presence of androgen supplementation). It should be noted that the shRNA to block YES expression, the most highly expressed SFK isoform, not only did not inhibit tube formation, but had a small stimulatory effect, suggesting YES negatively regulated caveolae-mediated signaling in HUVEC. FYN and LYN were the predominant SFK isoforms involved in mediation of signaling associated with "angiogenesis" in the HUVEC endothelial tube formation assay. During the last year of the project, these studies will be repeated with freshly established primary cultures of human prostate endothelial cells to validate the relative importance of the individual SFK isoforms as potential druggable targets to inhibit angiogenesis in human prostate cancer.

b) **Inhibition by SFK-inhibitors of angiogenesis in primary xenografts of human prostate**:

Based upon the *in vitro* studies that demonstrated SFK inhibitors inhibit directly, without toxicity, the angiogenic activity of HUVEC, preliminary studies of the effect of broad spectrum receptor tyrosine kinase (RTK) inhibitors on angiogenesis in primary xenografts of human prostate tissue were conducted. In the ensuing year, these studies will be extended to evaluate the *in situ* effect of the shRNAs specific for the individual SFK isoforms on angiogenesis by administration of lentivirus expressing the shRNA in the *in vivo* primary xenograft model.

For these studies, prostate tissue was transplanted into immuno-compromised mouse hosts that had been pre-implanted with sustained release testosterone pellets, and treatment of the hosts with the RTK inhibitor begun on the day after transplantation of the prostate tissue. Microvessel density in the xenografts was evaluated on Day 14 post-transplantation. Hosts were treated with four different anti-angiogenic agents that are in routine clinical use: a) avastin – a humanized antibody for VEGF; b) ABT-510 – a thrombospondin mimetic peptide; and c) two broad spectrum RTK-inhibitors, Sunitinib and Sorafinib. The dose and route for the anti-angiogenic agents were: ABT510 - 30mg/kg (i.p.) 2x daily (a gift of Abbott Laboratories); Avastin - 10mg/kg every 3 days (i.p.); Sunitinib - 10mg/kg daily (gavage); Sorafenib - 10mg/kg daily (gavage). While avastin and the thrombospondin-mimetic had marginal effects on angiogenesis by the endogenous human vessels in the primary xenografts, both of the RTK inhibitors, presumably through direct effects on SFK members in the endothelial cells, significantly inhibited angiogenesis in the primary xenograft model. We inferred that the inhibitory activity was not due to action on the VEGF-R since avastin did not inhibit angiogenesis as a monotherapy. Therefore, specific targeting of the appropriate SFK(s) in the endothelial cells of the human prostate that are associated with angiogenic activity could represent a novel prostate specific therapeutic approach for management of localized prostate cancer.



Key Research Accomplishments

- Development and validation of *in vitro* and *in vivo* human models for evaluating the role of SFKs in androgen-mediated uptake/transport/signaling/angiogenesis in human prostate endothelial cells;
- Demonstration that disruption of SFK-dependent signaling in human prostate endothelial cells can inhibit

angiogenesis both *in vitro* (primary cultures) and *in vivo* (primary xenografts).

Reportable Outcomes
None

Conclusion
The project is progressing toward the stated goals with no obstacles encountered to date.

References
This work was presented as both a poster and a podium presentation at the CDMRP Innovative Minds in Prostate Cancer Research (IMPaCT Conference) in Orlando, Fl in March 2011.

Appendices
None